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Carcinogenic Potential of Arsenic Compounds in Drinking Water

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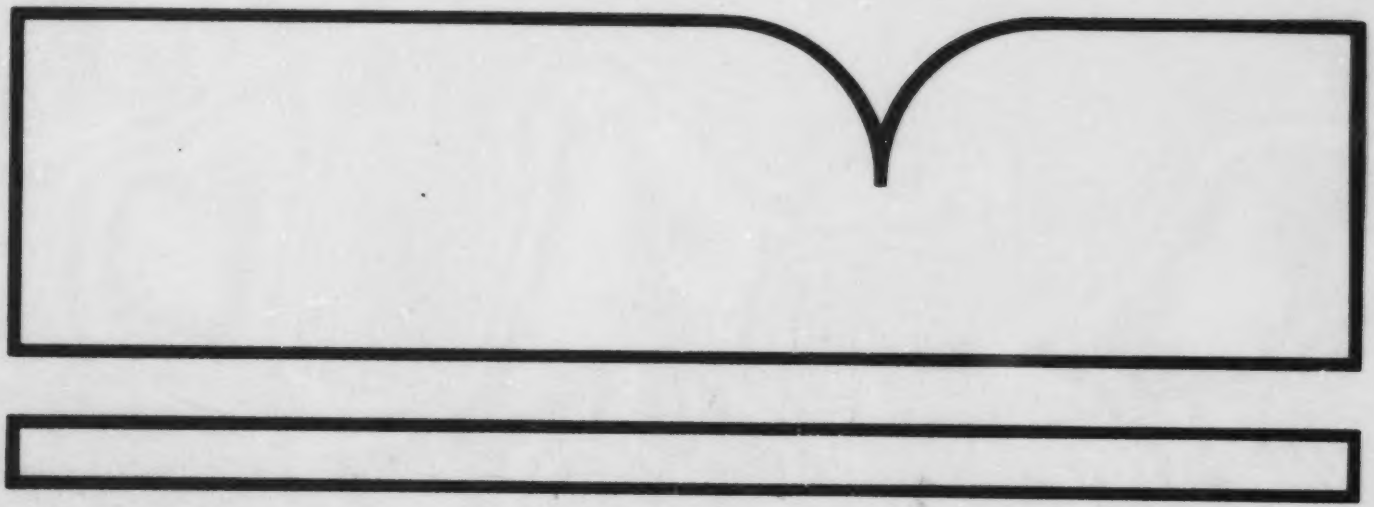

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CARCINOGENIC POTENTIAL OF ARSENIC COMPOUNDS IN DRINKING WATER

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16. ABSTRACT

The primary objective of this research project was to determine whether arsenite (AsIII), arsenate (AsV), dimethylarsinic (DMA) and monomethylarsonic acid (MA) were initiator carcinogens and/or promoters of DENA-initiated tumors in the rat liver.

The maximum tolerated doses (MTD) of the arsenics to be used in this study were determined by treating male Wistar rats in the drinking water increasing concentrations ranging from 10ppm-2560ppm for 7 weeks, depending upon the arsenic studied. The apparent MTD determined were: MA, 800ppm; AsIII, 160ppm; AsV, 160ppm; and DMA, 80ppm. The calculated LD_{50} were DMA, 98.5ppm; AsIII, 264.8ppm; AsV, 294.5ppm; and MA, 1160.1ppm.

To determine whether the arsenic compounds had an initiator or promoter activity, male Wistar rats were partially hepatectomized, treated with a single dose of diethylnitrosamine (30mg/kg i.p.) and on day 7 started treatment with the MTD of each arsenic for 7, 25 and 43 weeks in the drinking water. This was the promoter protocol. The initiator protocol did not include the diethylnitrosamine treatment. There were no significant numbers of animals with tumors in the liver in any of the arsenic treated animals as compared to their controls at the three time periods of treatment with either the initiation or promotion protocols.

However, in the promotion protocol there was significant increase ($p < 0.05$) in the number of animals exhibiting tumors in the kidney in the AsIII promoted group, 7/10, as compared to the DENA controls, 2/9 when treated for 25 weeks. In both protocols AsIII treated animals showed a significant decrease in thymus gland weights. This was suggested to be a contributory factor in the renal tumorigenesis observed in this study.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The complexities of environmental problems originate in the deep interdependent relationships between the various physical and biological segments of man's natural and social world. Solutions to these environmental problems require an integrated program of research and development using input from a number of disciplines. The Health Effects Research Laboratory, Research Triangle Park, NC and Cincinnati, OH, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. Wide ranges of pollutants known or suspected to cause health problems are studied. The research focuses on air pollutants, water pollutants, toxic substances, hazardous wastes, pesticides, and nonionizing radiation. The laboratory participates in the development and revision of air and water quality criteria and health assessment documents on pollutants for which regulatory actions are being considered. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of environmental regulatory decisions involving the protection of the health and welfare of all U.S. inhabitants.

In this report the results are presented from three years of study to determine whether arsenite (AsIII), arsenate (AsV), dimethylarsinic acid (DMA), and monomethylarsonic acid (MA) were initiator carcinogens and/or promoters of tumors in the rat liver. This work was initiated to gain data that might clarify some of the apparent contradiction between human and animal data and provide the basis for arriving at more realistic estimates of the carcinogenic risk that is associated with the various forms of arsenic found in drinking water.

F. Gordon Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

The primary objective of this research project was to determine whether arsenite (AsIII), arsenate (AsV), dimethylarsinic (DMA) and monomethylarsonic acid (MA) were initiator carcinogens and/or promoters of DENA-initiated tumors in the rat liver.

The maximum tolerated doses (MTD) of the arsenics to be used in this study were determined by treating male Wistar rats in the drinking water increasing concentrations ranging from 10ppm-2560ppm for 7 weeks, depending upon the arsenic studied. The apparent MTD determined were: MA, 800ppm; AsIII, 160ppm; AsV, 160ppm; and DMA, 80ppm. The calculated LD_{50} were DMA, 98.5ppm; AsIII, 264.8ppm; AsV, 294.5ppm; and MA, 1160.1ppm.

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However, in the promotion protocol there was significant increase ($p < 0.05$) in the number of animals exhibiting tumors in the kidney in the AsIII promoted group, 7/10, as compared to the DENA controls, 2/9 when treated for 25 weeks. In both protocols AsIII treated animals showed a significant decrease in thymus gland weights. This was suggested to be a contributory factor in the renal tumorigenesis observed in this study.

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SECTION 1

INTRODUCTION

The relationship between arsenic exposure and the development of cancerous lesions in humans has been reviewed by IARC (1973), Yeh (1973), Zaldivar (1974), Bencko (1976), Committee (1977), and Wildenberg (1978). They cite many clinical and epidemiological observation which indicate that when humans are exposed to arsenic, be it dietary, medical, environmental or occupational exposure, neoplasms of their liver, lung, skin, lymphatic and hematopoietic tissues result. Hueper (1963) referred to arsenic as "after coal soot, the oldest known human carcinogen". Dinman (1974) placed arsenic, along with mustard gas, in a "definitely carcinogenic" category based on epidemiological evidence for "carcinogenic potential".

However, not one of the 46 arsenic exposure experiments listed in the "Survey of Compounds Which Have Been Tested for Carcinogenic Activity," conclusively demonstrated arsenic's carcinogenic potential (Hartwell, 1951; Shubik and Hartwell, 1957). Nor have any recent studies demonstrated arsenic carcinogenesis in animals (Wildenberg, 1978). So, even though epidemiological evidence shows arsenic exposure is related to human cancers, attempts to induce cancer experimentally with arsenic compounds have not been successful. Even in the short-term mutagenicity tests, arsenic compounds (like other metals) have given negative results (Wildenberg, 1978).

There are several reasons why the data from experimental animals have not demonstrated a positive correlation between arsenic exposure and carcinogenesis, two of these are:

(1) The route of administration was via the diet or by application to the skin. Arsenic, unlike other skin carcinogens, does not exert its action by direct contact. There are no documented cases of human skin cancers following the external application of medicinal arsenicals, nor is there evidence that arsenic in food acts as a human carcinogen. Man's primary routes of arsenical exposure which resulted in cancer have been from ingestion in aqueous and alcoholic (in wines) solutions and inhalation (Committee, 1977). Schrauzer et al. (1978) have reviewed the reports which indicate inorganic arsenic compounds have different toxicities and interactions with selenium if animals drink as opposed to eat their arsenic.

(2) In general, only a single concentration of only one arsenic compound was tested and the maximum tolerated dose (MTD) was not determined which lead to poor animal survival and there being too few animals and tumors produced per experimental treatment group to allow a definitive statement concerning arsenic carcinogenesis to be made. Even if the number of animals per test were increased substantially, only those

chemicals which cause high tumor incidence would readily be detected as carcinogens. But observations in humans indicate arsenicals are probably weak carcinogens (Wildenberg, 1978).

Therefore, what is needed are bioassays which have greater sensitivity to arsenic than do either the classical long-term feeding studies or the short-term microbiological mutagenicity tests. We propose to explore the use of an assay which uses a two-stage carcinogen treatment to demonstrate experimentally the carcinogenic potential of four different arsenic compounds.

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

The experimental design for this study which was the broad spectrum initiation-promotion protocol developed at HERL Cincinnati was effective in determining whether a specific arsenic compound had an initiator carcinogen potential or a promoter potential. Although the procedure was designed for testing potential initiators and/or promoters in the liver, we believe it can be used in studying potential agents in the kidney as our results have shown.

We have demonstrated that the chronic toxicity of arsenic compounds when given in the drinking water can not be predicted from acute toxicity studies. We have shown that an organic arsenic such as dimethylarsinic acid was as toxic as arsenite or arsenate when administered chronically in the drinking water. According to acute toxicity studies arsenite and arsenate are methylated to dimethylarsinic acid which is suppose to be an inactive metabolite. There appears to be a discrepancy between the results of the acute toxicity study and the results. The results of this study showed that metabolism would result in an equitoxic metabolite. Thus, it is clear that the toxicity of these compounds have to be reevaluated under chronic exposure conditions in order to determine the effect of metabolism on steady-state levels of the metabolites.

We have demonstrated that arsenite is a promoter in the kidney. Chronic arsenite treatment for 25 weeks promoted DENA-initiated tumors in the kidney. Thus, arsenite may now be used as a known promoter in the kidney in order to test for potential initiator carcinogens in the kidney.

We have also demonstrated that arsenite decreases the thymus weight which is suggestive of a decrease in the immune response. Thus, this immunosuppressive effect may be a contributory factor in the carcinogenesis process.

Because these experiments were performed with partial hepatectomy, it must be determined that this surgical procedure did not contribute to the promotion effect of arsenite. Thus, we recommend that experiments be done on intact animals under the same conditions of dosing. We would anticipate that the treatment time will be longer and the time for appearance of renal tumors will also be extended.

Since the arsenite concentration used in this study was the maximum tolerated dose, we recommend that a dose-response study be done to determine at what level arsenite no longer has promoter activity. This would be important for setting safe drinking water standards.

Because of the discrepancies we found between our results obtained from our chronic dosing in the drinking water as compared to the results found in

the literature from acute toxicity studies, we recommend that metabolism of the different arsenics be studied *in vivo* chronic dosing in the drinking water. This would also be important in setting safe drinking water standards.

Finally, we recommend that the immunosuppressive effect of arsenite be studied further which might shed some light on the role of arsenite in the chemical carcinogenesis process.

SECTION 3

THE MAXIMUM TOLERATED DOSE (MTD) AND THE LD_{50} OF ARSENIC COMPOUNDS IN WISTAR RATS EXPOSED TO ARSENIC COMPOUNDS IN THE DRINKING WATER FOR 7 WEEKS

INTRODUCTION

Although arsenic has been known to be toxic for many years and doses for acute toxicity have been published for experimental animals, data for MTD in animals chronically exposed to arsenic in the drinking water are not available, in particular, that for organic arsenics such as monomethylarsonic acid (MA) and dimethylarsinic acid (DMA) because the acute toxicity studies showed that these arsenics were not toxic.

Thus, the purpose of this study was to determine the maximum tolerated dose (MTD) for each species of arsenic studied in this investigation, namely, arsenite (AsIII), arsenate (AsV), monomethylarsonic acid (MA) and dimethylarsenic acid (DMA) when they were chronically administered to experimental rats in the drinking water for a period of seven weeks.

MATERIALS AND METHODS

Animals

Male Wistar rats (60-80 g), obtained from Simonsen Laboratories, Inc. (Gilroy, CA) were randomly divided into groups of five rats each and given access to distilled water containing various parts per million (ppm) arsenic as either sodium arsenite (AsIII); sodium arsenate (AsV); methylarsonic acid, disodium salt (MA); or dimethylarsinic acid, sodium salt (DMA)for seven weeks. Each group was housed in individual gang cages and fed a standard laboratory chow. The weights of all test animals were recorded weekly. Gross observations were made during the test period. At 7 weeks the animals were sacrificed by guillotine and routine necropsy was performed.

LD_{50} Calculation

The doses of the toxicity data was expressed in concentration. The LD_{50} was calculated by the method of Reed-Munch (1938).

RESULTS

MTD and LD_{50}

The weekly weight gain data for rats receiving the various arsenic treatments are presented in Figure 3-1. All of the arsenic compounds caused a reduction of weight gained as compared to the controls at nearly all doses of the different arsenic species tested. The effect appeared to be greatest with

DMA> AsIII> AsV> MA. At 160 ppm of MA there appeared to be an increase in the weight gained over the 7-week treatment period. The apparent MTD determined according to the criteria stated in the Methods were: MA, 800 ppm; AsV, 160 ppm; AsIII, 160 ppm; and DMA, 80 ppm.

The calculated LD_{50} values are shown in Table 3-1. The data suggest that DMA was the most toxic followed by AsIII and AsV which were more toxic than MA. These results contradict the toxicity data in the literature obtained from acute exposure to arsenic in which inorganic arsenics (AsIII and V) were nearly ten times more toxic than DMA (Committee, 1977).

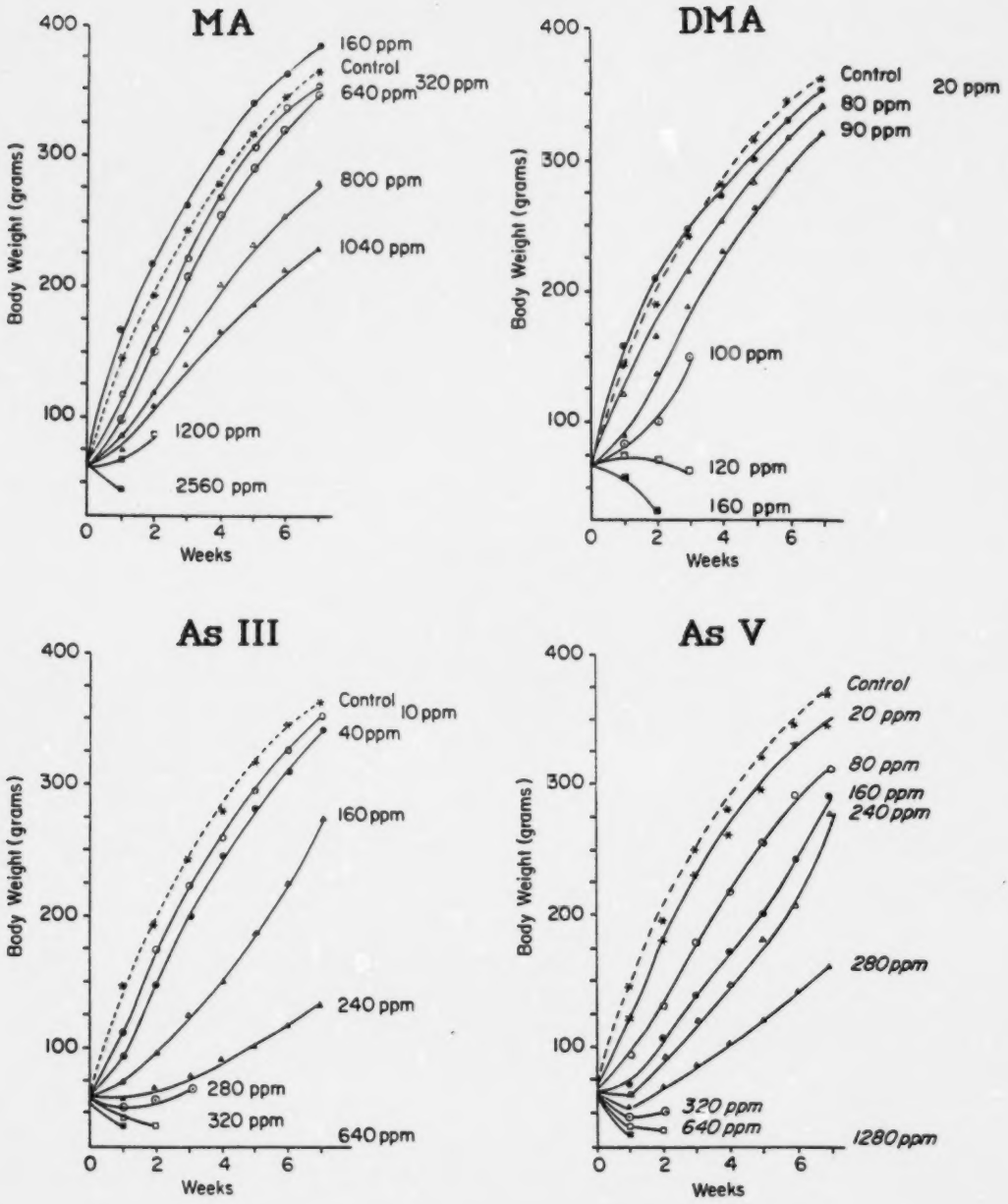

Figure 3-1. The Effect of 7 Week Treatment of Different Arsenic Compounds on Growth of Wistar Rats.

Table 3-1. The Calculated LD_{50} of Arsenic Compounds in Male Wistar Rats After Chronic Exposure of These Xenobiotics Via the Drinking Water for 7 Weeks.

Arsenic Compound	Calculated LD_{50} (in ppm)	Ranges of Concentrations Tested (in ppm)
DMA	98.5	20 - 320
AsIII	264.8	10 - 640
AsV	294.5	20 - 1280
MA	1160.1	160 - 2560

Gross Observations of Toxicity

At doses below the MTD the only general observable effect of the arsenic treatment was the slower growth rate, except at the lowest dose of MA where there appeared to an increase in growth rate, especially in the first several weeks. At the MTD to LD_{50} doses, in addition to the slower growth rate, the animals were more excitable during the first week of treatment. The animals were more difficult to catch and had a tendency to jump from the cages. After the initial excitement stage, however, the arsenic treated animals behaved like the control by remaining huddled together even when the cage doors were opened.

As the dose of the inorganic arsenics and MA were increased, the rats became depressed. Their coats became more rough and the hair around the facial area became thin. Watery diarrhea, anorexia and dehydration were seen in rats receiving LD_{50} doses or more in arsenic treatment. The severity of the symptoms appeared to be directly related to the dose. Those rats who died from the inorganic or MA treatment exhibited symptoms of stupor and coolness in the extremeties, poor coat condition and morbidity for an extended period before death. Even at LD_{50} doses the animals would live for one to two weeks before death. Those rats receiving DMA, however, exhibited only a slower growth rate and slight diarrhea before death. Generally, the DMA treated rats lived longer before death than those treated with comparable toxic doses of the inorganic arsenics or MA.

Necropsy

The rats surviving LD_{50} doses or more of inorganic arsenic and MA treatment had slightly enlarged hearts and adrenal glands and a smaller thymus gland than control rats. The most noticeable change was the increased size of the caecum; which could be three to five times larger than the control rat's caecum. The blood vessels on the sides of the caecum were usually prominent. The caecum contents were usually dark, fluid and filled with foul-smelling gas bubbles. This odor could be easily detected upon first opening the peritoneal cavity. At the highest doses, the entire intestinal contents consisted of a foul-smelling, bright yellow liquid. The kidneys were gray-colored, soft, with a mottled appearance and exhibited a sharp demarkation between the

cortical and medullar regions. In rats receiving the MTD to LD_{50} doses, the above changes were absent or only present to a slight degree. In many of the rats receiving MTD and lower doses of the arsenics, the thymus and adrenal glands were enlarged. The tissues of rats receiving MTD plus doses were darker and softer than those of the untreated rats. The muscles of rats receiving LD_{50} plus doses were more flaccid than those of the control rats. The severity of these conditions was dose related. The blood of arsenic treated rats was very dark and did not flow as rapidly as the blood from control animals.

The control animals all exhibited numerous and prominent/spontaneous polyplike growths on the external walls of their intestine. Rats receiving MTD plus doses of the arsenic compounds rarely had any of these polyps. Rats receiving less than the MTD doses of the arsenicals had fewer and less prominent polyps than did the control rats.

DISCUSSION

The data from our MTD and LD_{50} studies showed that the most toxic of the arsenic compounds studied in this investigation was DMA followed by AsIII> AsV> MA. These results are quite different from that found from acute toxicity studies where the inorganic arsenics, AsIII and AsV, were much more toxic than DMA. These data could be influenced to some degree by differences in the amount of water intake. It appeared that the animals treated with inorganic arsenics were dehydrated as compared to the animals treated with organic arsenics, However, even if the inorganic arsenic treated animals drank 1/2 the amount of water as the DMA treated animals, this would result in near equal degree of toxicity but still in the direction of DMA in terms of the LD_{50}. In terms of the MTD the doses will be equal. Thus, prolonged exposure to these compounds in the drinking water revealed a different toxicity profile than that which would have been predicted from the acute toxicity studies by others. However, there was agreement with the literature that MA was the least toxic of the four arsenic species.

Some other differences in effects were also noted here which differed from data from acute toxicity studies. Usually it is accepted that AsIII is more toxic than AsV primarily because the trivalent form can bind to functional sulfhydryl groups (Pisciotto and Graziano, 1980), whereas the pentavalent form does not bind at these sites but might interfere with key biochemical reactions involving phosphate. However, in our studies AsIII and AsV appeared to be equitoxic in both the MTD and the calculated LD_{50} dose. So it is interesting to note here that the toxicity of arsenic was not necessarily dependent upon whether arsenic was in the trivalent or pentavalent form. Whether this was coincidental or not cannot be determined from our data, however, it is interesting to note that DMA is also a pentavalent arsenic.

Another difference noted was that the toxic signs observed with DMA treatment differed considerable from that observed with AsIII, AsV and MA treatments. Thus, these differences cannot easily be explained as differences between inorganic and organic arsenic toxicities.

Very often differences in toxicities could be explained by hepatic biotransformation. It has been generally accepted that the inorganic arsenics,

AsIII and AsV, are converted to the less toxic organic arsenics, MA, then subsequently to DMA, by the liver and then excreted via the urine. This explains the results of the acute toxicity studies in which the inorganic arsenics are much more toxic than the organic arsenics. However, in our studies which is a moderately chronic exposure study, the metabolite, DMA, is at least equitoxic as AsIII. Either AsIII and AsV were rapidly converted to DMA and the toxicities observed were due to DMA, or DMA and AsV at steady-state were converted to AsIII and that the toxicities observed were really due to AsIII. The latter is unlikely since the major urinary metabolites are the methylated metabolites. The former is also unlikely since the toxicity observed were different from that observed with AsIII, AsV and MA. Furthermore, MA at the concentrations given would have formed more DMA than with DMA treatment alone. Therefore, it should have been the most toxic, but instead it was the least toxic. Thus, our current knowledge of the biotransformation of arsenic compounds does not shed any light on the mechanism(s) of the toxicities involved in this study.

In summary, it is clear that data obtained from acute arsenic toxicity of these same compounds were different from our study. More studies have to be made under chronic exposure conditions in order to determine steady state blood and tissue levels of the various arsenic species and the interconversion of the different arsenic compounds. Finally, it is reasonably safe to conclude that the mechanisms involved in the toxicity of these arsenic compounds are much more complicated than heretofore imagined and are not easily differentiated into inorganic or organic arsenic toxicities but more than likely must be determined for each arsenic species.

SECTION 4

THE STIMULATION OF GAMMA-GLUTAMYLTRANSPEPTIDASE ACTIVITY IN RAT LIVER BY INORGANIC ARSENICS

INTRODUCTION

Gamma-glutamyltranspeptidase (GGTase) is a fetal enzyme present in neonatal rat liver. Its activity is decreased to a low level with age. Since its activity is relatively high in hepatic tumors, it is often used as an enzyme marker for identification of potential preneoplastic tissue in the liver (Solt and Farber, 1976, Peraino et al., 1978; Pitot et al., 1978). In these studies an initiation-promotion model for chemical carcinogenesis was utilized.

Although arsenic has been associated primarily with tumors found in the skin, lung, liver and lymphoid tissues of humans (Neubauer, 1947; Tseng et al., 1968; Lee and Fraumeni, 1969; Yeh, 1973; Ott et al., 1974; Tokudome and Kuratsune, 1976; Pinto et al., 1976; Mabuchi et al., 1979; IARC, 1980; Luchtrath, 1983), the demonstration of tumorigenesis by arsenic in experimental animals has been negligible (Ishinishi et al., 1977; Ivankovic et al., 1979). Thus, the liver was chosen as a test tissue to see if arsenic had a potential to stimulate or to maintain hepatic fetal GGTase activity, which might explain the possible role of arsenic as a component of the chemical carcinogenesis process.

MATERIALS AND METHODS

Animals

Five-week old Male Wistar rats obtained from Simonsen Laboratories, Inc. (Gilroy, CA) were randomly divided into groups of five rats each, housed in individual gang cages and given access to standard laboratory rat chow and to distilled water or distilled water containing 160ppm (elemental arsenic) of either sodium arsenite (AsIII) or sodium arsenate (AsV). The animals were sacrificed at predetermined times by guillotine. The liver tissue was quickly excised and prepared for histochemical and biochemical studies.

Biochemical Assay for GGTase Activity

Initially 1 g. of rat liver was removed from each animal and stored in a Revco deep freezer at -70°C prior to preparation of enzyme homogenate. The tissue was then thawed, minced and washed two times in a buffer solution containing 50mM phosphate and 0.25M sucrose. The tissue was then homogenized in 9 ml of the same buffer by a motor-driven homogenizer and stored frozen at -20°C before being assayed (usually 2-3 days). No loss of activity was observed with this procedure. Duplicate aliquots of 10-50 ul (0.2-1.0 mg protein) were incubated in a medium containing 4.6mM L-gamma-glutamyl-p-nitroani-

lide (Sigma Chemical Co. St. Louis, MO) as substrate according to the method described by Naftalin et al. (1969). The p-nitroaniline liberated is diazotized according to a modified Bratton-Marshall reaction (Bratton and Marshall, 1939). The absorbance of the pink dye was measured at 545nm on a Beckman Model 24 spectrophotometer. Specific activity was expressed as nmoles of p-nitroaniline released per minute per mg of protein.

Protein Determination

The protein content of the liver homogenates was determined by the method of Lowry (1951).

Histochemical Assay for GGTase Activity

The presence of GGTase activity was detected according to the method described by Rutenberg et al. (1969). Approximately a 2mm thick slice of liver (right lobe) measuring approximately 1cm x 2cm was removed from each rat and frozen by a continuous stream of carbon dioxide. The frozen tissue was transferred to an Ames Lab-Tek cryostat, sliced into 10 micron serial sections, placed on glass slides and incubated for one hour at 37°C in a freshly prepared medium containing 12.5 mg/100ml gamma-glutamyl-4-methoxy-2-naphthylamide (Bachem, Torrance, CA) as substrate. The glass slides were dried and mounted (Aqua Mount, Lerner Labs, New Haven, CT) for microscopic examination and scored for the presence of GGTase active foci.

The GGTase active foci were scored from 0 to 6, with a score of 0 being given when no readily apparent GGTase activity was present to the naked eye and a score of 6 being given for the maximum number of active foci observed. Standards were determined for each score and photomicrographed. The photomicrographs were used as reference for the scoring procedure.

Statistical Analysis

All comparisons were made by Student t test.

RESULTS

Effect of AsIII and AsV on GGTase Activity

The stimulation effect of AsIII and AsV on liver GGTase activity is shown in Tables 4-1 and 4-2. At 5 weeks old the control fetal enzyme activity was still somewhat high. It decreased in the control animals to a basal level at approximately 9 to 10 weeks of age. This high GGTase activity was increased in the AsIII and AsV treated animals after one week of treatment. By the second and third week of treatment, the stimulation was significantly higher than the controls both histochemically and biochemically. A significant increase in GGTase activity would probably be observed at an earlier time period, if the number of animals in each treatment group were increased. Although the stimulation appeared to be greater in the AsIII animals, the results were much more variable such that AsV stimulation was as effective.

Table 4-1. The Effect of Chronic AsIII and AsV Treatment on Hepatic GGTase Activity Determined Histochemically.

Time of Exposure	Average Score of GGTase Activity Control	AsIII Treatment	Std Error of the Means AsV Treatment
Days			
1	1.4 ± 0.5	1.4 ± 0.4	1.2 ± 0.3
2	1.2 ± 0.5	1.6 ± 0.3	2.4 ± 0.5
4	1.2 ± 0.2	1.7 ± 0.4	1.8 ± 0.4
Weeks			
1	1.2 ± 0.2	2.8 ± 0.7	3.8 ± 0.4
2	0.8 ± 0.2	4.2 ± 0.4*	4.0 ± 0.5*
3	0.6 ± 0.3	3.2 ± 0.4*	2.3 ± 0.4*
4	0.2 ± 0.1	4.3 ± 0.3*	2.0 ± 0.1*
5	0.1 ± 0.1	3.8 ± 0.5*	2.6 ± 0.4*
6	0.0 ± 0.0	3.2 ± 0.4*	2.0 ± 0.5*
7	0.0 ± 0.0	3.8 ± 0.3*	3.2 ± 0.2*

* These values were significantly different from the corresponding control values at $P < 0.05$ level as determined by Student t test.

Table 4-2. The Effect of Chronic AsIII and AsV Treatment on Hepatic GGTase Activity Determined Biochemically.

Time of Exposure	Mean GGTase Activity (in nmoles/mg protein/min) Control	AsIII Treatment	Std Error of the Means AsV Treatment
Days			
1	1.76 ± 0.48	1.77 ± 0.40	1.52 ± 0.28
2	1.05 ± 0.39	1.82 ± 0.33	1.21 ± 0.33
4		2.75 ± 0.68	1.07 ± 0.31
Weeks			
1	1.38 ± 0.29	3.93 ± 1.27	4.74 ± 1.80
2	0.59 ± 0.05	7.58 ± 1.86*	7.35 ± 3.01
3	0.61 ± 0.11	2.71 ± 0.53*	1.90 ± 0.52*
4	0.38 ± 0.04	6.31 ± 2.76	0.88 ± 0.24
5	0.29 ± 0.04	4.83 ± 1.78*	1.55 ± 0.36*
6	0.27 ± 0.03	4.88 ± 1.64*	1.58 ± 0.39*
7	0.31 ± 0.05	2.88 ± 0.75*	1.52 ± 0.15*

*These values were significantly different from the corresponding control values at $P < 0.05$ level as determined by Student t test.

The Effect of Long-Term Exposure to AsIII and AsV

The effect of prolonged exposure to AsIII and AsV up to 24 months is shown in Table 4-3. Although the GGTase activity decreased with age initially, after 15 and 20 months the GGTase activity increased dramatically in the control animals. This observation was contrary to the accepted notion that GGTase activity is a fetal enzyme. Interestingly AsIII and AsV treatment for 15 and 24 months also led to a corresponding significant increase in GGTase activity.

Table 4-3. The Effect of Prolonged AsIII and AsV Treatment on Rat Hepatic GGTase Activity.

Time of Exposure (in months)	Mean Specific Activity of GGTase		
	Control	AsIII Treatment	AsV Treatment
6	0.52 ± 0.17 (14)	4.32 ± 2.92 (3)	2.83 ± 1.83 (4)
10	0.63 ± 0.15 (16)	5.22 ± 0.94* (13)	4.29 ± 0.92* (14)
12	0.57 ± 0.21 (10)	2.47 ± 0.51* (9)	1.33 ± 0.62 (5)
15	1.60 ± 0.43 (11)	3.63 ± 0.63* (22)	4.66 ± 1.16* (16)
24	3.35 ± 0.71 (16)	12.45 ± 2.55* (7)	11.05 ± 2.84* (5)

Specific activity of GGTase is expressed in nmoles product formed/mg protein/min ± standard error of the means (number of animals).

* These values are significantly different from their corresponding control values at $P < 0.05$ as determined by the Student *t* test.

The treatment dose for both AsIII and AsV was 160ppm in the drinking water.

The Effect of Organic Arsenics on GGTase Activity

We did not observe any significant increases in GGTase activity in any of the DMA- or MA-treated animals at any of the time periods studied (data not shown). In some of the DMA treated groups there appeared to be some increases but they were not statistically significant.

The Effect of Time on GGTase Stimulation By AsIII and AsV

The results of experiments designed to determine whether time was a critical factor in the GGTase stimulation by the inorganic arsenics are shown in Table 4-4. The effects were examined both histochemically and biochemically. The results showed that if the animals were treated with either AsIII or AsV for 3 weeks and then left on water alone for two weeks, the GGTase activity still remained significantly elevated over controls. Even at 9 weeks on water

alone, AsV treatment still maintained significantly increased GGTase activity. With AsIII treatment the activity was increased but it was not statistically significant due to the variability and the small number of animals in the treatment group. If they were left on water for 22 weeks, then the GGTase activity declined to control levels.

However, if the animals were initially left on water for 3 weeks before treating them with AsIII or AsV for 3 weeks, then the GGTase activity was no longer stimulated. Thus, GGTase in livers from 8 week old rats were not stimulated by 3 week treatment with AsIII or AsV in the drinking water.

The Effect of Phenobarbital on the Stimulation of GGTase Activity by AsIII and AsV

Since it is well established that diethylnitrosamine-initiated rats fed 0.05% phenobarbital (PB) in the diet cause foci of GGTase activity in the liver, experiments were designed to test whether PB would enhance the inorganic arsenic stimulation of GGTase activity in the liver. The results are shown in Table 4-4. The data showed that PB did not stimulate GGTase activity after 12 or 25 weeks of treatment but it did appear to enhance the inorganic arsenic stimulation of the enzyme after 22 weeks of PB treatment. There was a significant increase in both AsIII and AsV treatment groups in the presence of PB as compared to the PB control.

Table 4-4. The Characterization of the Stimulation of Hepatic GGTase by AsIII and AsV.

Experimental Conditions		GGTase Activity Histochemical	GGTase Activity Biochemical
Control H_2O for	5 wks	1.0 ± 0.24 (6)	0.29 ± 0.04 (6)
AsIII 3 wks, followed by H_2O	2 wks	2.2 ± 0.37 (5)	0.95 ± 0.23 (5)*
AsV 3 wks, followed by H_2O	2 wks	2.0 ± 0.54 (5)	0.84 ± 0.19 (5)*
Control H_2O for	12 wks	0.5 ± 0.34 (6)	0.31 ± 0.05 (6)
AsIII 3 wks, followed by H_2O	9 wks	2.0 ± 0.58 (3)	1.27 ± 0.62 (3)
AsV 3 wks, followed by H_2O	9 wks	2.6 ± 0.40 (5)	1.22 ± 0.38 (5)*
Control H_2O for	25 wks	1.2 ± 0.33 (14)	0.52 ± 0.17 (14)
AsIII 3 wks, followed by H_2O	22 wks	1.2 ± 0.37 (5)	0.35 ± 0.06 (5)
AsV 3 wks, followed by H_2O	22 wks	2.3 ± 0.75 (4)	0.86 ± 0.24 (4)
H_2O 3 wks, followed by AsIII	3 wks	0.3 ± 0.21 (6)	0.35 ± 0.05 (6)
H_2O 3 wks, followed by AsV	3 wks	0.2 ± 0.17 (6)	0.32 ± 0.05 (6)
PB for	12 wks	1.3 ± 0.33 (3)	0.60 ± 0.27 (3)
AsIII 3 wks, followed by PB	9 wks	2.8 ± 0.48 (4)	1.38 ± 0.58 (4)
AsV 3 wks, followed by PB	9 wks	2.7 ± 0.33 (3)	1.89 ± 0.49 (3)
PB for	25 wks	1.0 ± 0.42 (8)	0.69 ± 0.22 (8)
AsIII 3 wks, followed by PB	22 wks	2.8 ± 0.29 (10)	2.00 ± 0.56 (10)*
AsV 3 wks, followed by PB	22 wks	2.6 ± 0.75 (5)	2.23 ± 0.70 (5)*

* These values were significantly different from the corresponding control

values at $P < 0.05$ level as determined by the Student *t* test.

GGtase activity is expressed as nmoles product formed/mg protein/min.

Treatment dose for both AsIII and AsV was 160ppm in the drinking water.

Treatment dose for PB was 0.05% in the food pellet.

The number within the parenthesis ( ) denotes the number of animals in each treatment group.

Site of GGTase Activity

The fetal GGTase activity present in the 5 week old rat was observed to be located primarily in the periportal area when assayed histochemically. The increased GGTase activity due to the effects AsIII and AsV was also shown to be localized in the periportal area involving several layers of cells. There appeared to be no histochemically significant difference detected between the enhanced GGTase activity due to inorganic arsenic treatments or that due to PB alone.

DISCUSSION

It has been demonstrated that the enzyme activity of hepatic GGTase, a fetal enzyme, is elevated in what are suggested to be preneoplastic lesions in the liver (Solt and Farber, 1976; Peraino et al., 1978; Pitot et al., 1978). The purpose of this study was to determine whether certain arsenic compounds which have been associated with tumors, primarily in the skin and lungs but also in the liver, might have a potential to stimulate this enzyme. The results of this study demonstrated that AsIII and AsV administered chronically to 5 week old male rats in the drinking water significantly elevated hepatic GGTase activity.

This observation was suported by both histochemical and biochemical data. The time course of this effect was statistically significant within two weeks. The data was somewhat complicated by the fact that the enzyme activity was elevated initially in the 5 week old rats which took several weeks before it declined to a basal level. Otherwise, the increase probably would have been observed at an earlier time frame. With continuous presence of the arsenic the elevated activity was maintained for as long as two years.

However, it was clear that a critical concentration of AsIII and AsV was not necessary for the stimulation of the enzyme directly because 9 weeks after the arsenic treatment was discontinued, the GGTase activity was still elevated. This would suggest a genetic mechanism in which *de novo* synthesis of the enzyme occurred rather than a direct effect of AsIII or AsV as an explanation for the elevated GGTase activity. It also suggested that the turnover rate of the enzyme was relatively slow.

The mechanism by which these inorganic arsenics could have caused an increase in biosynthesis of the enzyme is not very clear, since AsIII is in the trivalent form and AsV is in the pentavalent form. However, in our laboratory we have observed that liver homogenates rapidly oxidized AsIII to AsV, but AsV was not easily reduced to AsIII (unpublished results). Therefore, it would seem reasonable to think that both arsenics are exerting their effects through a single common mechanism via AsV. An argument against this idea is that the stimulatory effect observed was generally slightly greater with AsIII than with AsV.

The chemical properties of AsV is very similar to that of the phosphate ion and biochemically it has been demonstrated to interfere with phosphorylation reactions (Mitchell et al., 1971). Therefore, it is possible that arsenate might inhibit the phosphorylation of a key nuclear protein which normally represses the gene necessary for the synthesis of GGTase in the adult liver. The derepression effect of arsenate allows for de novo synthesis of GGTase, leading to the increased GGTase activity observed in this study with AsIII and AsV.

Finally, the ability of AsIII and AsV to stimulate hepatic GGTase activity suggests a role for these agents as potential initiators and/or promoters of carcinogenesis. Since arsenic has been implicated as a carcinogen in humans for a number of years, the results of this study might contribute to the overall answer of its role in human carcinogenesis.

SECTION 5

THE INITIATOR POTENTIAL OF ARSENITE AND ARSENATE IN PARTIALLY HEPATECTOMIZED RATS WHEN PROMOTED BY PHENOBARBITAL

INTRODUCTION

The rat liver foci bioassay is a protocol that has been developed for the evaluation of carcinogenic potential of many different chemically unrelated substances. This bioassay for experimental hepatocarcinogenesis can be divided into several operational stages. Initiation and promotion represented the earliest stages of this mode. The initiation stage occurs by exposure of the organism to a known carcinogen, initiator carcinogen (IC), which produces a genotoxic alteration of cell(s) in the normal tissue. This is usually a single exposure. The promotion stage starts by administering multiple doses of a promoter which either selectively enhances the growth of the initiated cells or inhibits growth of normal cell population. Partial hepatectomy increases the sensitivity of the tissue to the chemical and decreases the time required for the bioassay.

Hepatocytes possessing altered enzymatic activity and altered cellular metabolism are used as early indicators of carcinogenesis. ATPase-negative, G-6-Pase-negative and GGTase-positive activities have been used as phenotypic markers of preneoplastic tissue.

Previous arsenic studies in non-hepatectomized rats (Section 4) demonstrated that AsIII and AsV stimulated GGTase activity in rat livers. Secondly, phenobarbital, a known promoter, appeared to enhance the inorganic arsenic stimulation of GGTase activity. Therefore, the purpose of this study was to test the potential of AsIII and AsV as initiator carcinogens, utilizing the 2 stage Broad Spectrum Protocol developed by HERL Cincinnati and using phenobarbital as the promoter.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 70-100g were 2/3 partially hepatectomized (PH) and 18-24 hours later were exposed to arsenic in their drinking water for a period of 3 days. The AsIII and AsV doses were 160ppm (MTD) and 320ppm. The higher dose was given in order that the concentration of As in the plasma and liver would be comparable to doses in animals exposed to 160ppm As for one week. Previous experiments indicated that 1 week was the minimum period of time necessary for GGTase stimulation at 160ppm. Animals were then given 0.05% phenobarbital food pellets (Teklad Test Diets) ad libitum 7 days after the PH and continued for 7 weeks. The animals were sacrificed by guillotine; livers were dissected out and processed as previously described in Section 4;

and routine necropsy was performed.

Nodule Formation

Nodule formation was graded according to the following criteria: +, one white or grey-white spot present; ++, several spots present on a single liver lobe; +++, one or more spots present on different liver lobes; ++++, tumor present rather than a nodule on any or all of the liver lobes. The degree of nodule formation was summed for each condition and divided by the number of animals with nodules.

GGTase Assay

The methodology for the histochemical and biochemical assays described in Section 4 was used in this study.

Broad Spectrum Protocol

The protocol developed by HERL Cincinnati was used which was a variation of the rat liver foci bioassay. The procedure involved partial hepatectomy of the rat which was followed in 18-24 hrs by exposure to the initiating carcinogen. One week following the PH a multiple dosing schedule of the promoter was given for a predetermined time.

RESULTS

Effect of Arsenic on Hyperplastic Nodule Formation

The effects of AsIII and AsV as initiator carcinogens on hyperplastic nodule formation are shown in Table 5-1. The results were variable, and no definitive trends were observed.

Table 5-1. The Effect of AsIII and AsV on Hyperplastic Nodule Formation in Livers From DENA-Initiated PB-Promoted Wistar Rats.

Experimental Condition	n	No. Animals with Nodules	Degree of Nodule Formation
AsIII (160ppm)	3	3	+
AsIII (160ppm) + PB	5	0	-
AsIII (320ppm) + PB	3	3	+++
AsV (160ppm)	3	1	+
AsV (160ppm) + PB	2	1	++
AsV (320ppm) + PB	3	0	-

All animals in this study were partially hepatectomized.

Effect of Arsenic on GGTase Activity

The effect of AsIII and AsV as initiator carcinogens on GGTase activity

are shown in Table 5-2. Again the data were variable with no apparent significant differences between the different treatment groups.

Table 5-2. The Effect of AsIII and AsV on GGTase Activity in Livers From DENA-Initiated PB-Promoted Wistar Rats.

Experimental Conditions	n	GGTase Activity	
		Histochemical	Biochemical
AsIII (160ppm)	3	1.5 ± 0.75	0.98 ± 0.43
AsIII (160ppm) + PB	5	2.0 ± 0.76	1.54 ± 0.87
AsIII (320ppm) + PB	3	1.5 ± 0.40	0.75 ± 0.11
AsV (160ppm)	4	2.0 ± 0.40	1.14 ± 0.57
AsV (160ppm) + PB	3	2.3 ± 0.18	1.19 ± 0.30
AsV (320ppm) + PB	3	2.6 ± 0.30	0.95 ± 0.17
DENA + PB	5	3.4 ± 0.71	3.95 ± 1.81

GGTase activity is expressed as mean score ± std error of the means or nmoles product formed/mg protein/min ± std error of the means.

All animals in this study were partially hepatectomized.

DISCUSSION

The results of this study was variable and no definite conclusions could be made. There were some limitations in the experimental design which should be noted. It is likely that the promotion period was probably not long enough because if the inorganic arsenics are initiator carcinogens, then they would probably be very weak as compared with DENA. If this is the case, then the initiator exposure time should have been longer and the promoter given at a later time commensurate with the arsenic exposure.

We concluded that the preliminary data in this study did not suggest that the inorganic arsenics were likely to be initiator carcinogens. In addition prior studies in other laboratories failed to demonstrate it also. Thus, the more likely role for these compounds would be as a promoter.

SECTION 6

PROMOTER POTENTIAL OF ARSENIC COMPOUNDS IN PARTIALLY HEPATECTOMIZED DENA-INITIATED RATS: I. The Effects in the Liver of Wistar Rats Chronically treated With Arsenic in the Drinking Water for 7 Weeks.

INTRODUCTION

From our previous study (Section 5) it was shown that chronic exposure of rats to inorganic arsenics, AsIII and AsV, stimulated hepatic GGTase activity. It was suggested that this increase in GGTase activity in the liver was due to de novo synthesis of the enzyme.

Recently, it has been suggested by several investigators that an elevated GGTase activity in the liver might be associated with preneoplastic lesions or foci (Solt and Farber, 1976; Peraino et al., 1978; Pitot et al., 1978). These investigators used the two stage liver initiation-promotion model, the initiator being diethylnitrosamine (DENA) and the promoter being phenobarbital (PB). In these studies the PB promotion resulted in discrete areas or foci of GGTase activity which were suggested to be preneoplastic. With time these foci are supposed to result in liver tumor(s).

Since we found that inorganic arsenics increased GGTase activity in post-fetal liver tissue and arsenic has long been associated with tumors in humans, we decided to test whether arsenic would also act like PB in partially hepatectomized DENA-initiated rats and cause these foci of GGTase activity to form and eventually result in hepatocellular carcinoma(s).

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 70-100g. were partially hepatectomized (PH) and injected i.p. 18-24 hrs later with DENA (Eastman Kodak) at a dose of 30mg/kg. On day 7 the animals were treated to one of the following: 0.05% phenobarbital (PB) in the diet, arsenite (AsIII) or arsenate (AsV) present in the drinking water at a level of 160ppm, or organic arsenicals, DMA and MA, present in the drinking water at a concentration of 80ppm and 800ppm, respectively. Each group contained a minimum of five animals. At the end of 7 weeks of treatment with PB or the arsenics the animals were sacrificed by guillotine and routine necropsy performed. Liver samples were removed for GGTase assay.

GGTase Assays

Liver tissues were prepared and histochemical and biochemical assays were performed for GGTase activity as previously described.

Scoring of Hyperplastic Nodules

See Materials and Methods of Section 5.

Histology

At autopsy the liver was removed and a portion of it was placed in buffered formalin (Fixx It, Lerner Labs, New Haven, CT). The tissue was dehydrated in denatured alcohol (5% isopropyl alcohol and 95% ethanol and xylene); embedded in a Paraplast:Paraplast Plus (60:40) medium; mounted on a AO microtome and sectioned at 6 micron thickness. The sections were transferred on to glass slides and stained with hematoxyln and eosin (American Histology Reagent Co. Stockton, CA). The glass slides were mounted with Adams Histoclad and examined by light microscope.

Histological Evaluation

The liver sections were evaluated for hyperplastic nodules, neoplastic nodules or hepatocellular carcinoma as described by Emmelot and Scherer (1980). A quality assurance procedure for this evaluation was developed by this laboratory in consultation with Dr. Mike Stedman of Pathology Associates, Inc.

RESULTS

Hyperplastic Nodule Formation

There was a strong correlation between inorganic arsenic treatment in PH animals and the presence of hyperplastic nodules on the liver surface as shown in Table 6-1. These white or gray-white growths were most often found on the medial-ventral surface of the right liver lobe. Qualitatively there appeared to be no difference between the effects of AsIII and AsV. The effects appeared to be dose dependent. DENA treatment by itself also appeared to result in nodule formation The effects of the organic arsenics were variable in this study.

Hepatic GGTase Activity

As was expected there was elevated GGTase activity associated with arsenic treatment as shown in Table 6-3. It appeared to be dose dependent and greater activity was observed in the DENA-initiated animals. It was noted at this time that perhaps the foci of GGTase activity observed histochemically was not as large as that noted by other laboratories. Since this particular experimental protocol was developed in the Sprague-Dawley and Fischer rat strains, it was possible that the Wistar rats were not as sensitive. Thus, a series of experiments were performed with Sprague-Dawley rats to verify this possibility (for Results see Section 7).

Histological examination of the GGTase activity did not show any specific foci of activity. It was extremely difficult to locate these foci because of the large background activity of the enzyme. Because of this difficulty in subsequent studies we decided to use another histochemical marker commonly used to identify altered cellular foci, namely, the deficiency of

Table 6-1. Formation of Hyperplastic Nodules in Livers from DENA-Initiated Rats Chronically Exposed to Inorganic Arsenics in the Drinking Water for 7 Weeks.

Experimental Condition	n	No Animals With Nodules	Degree of Nodule Formation
Sham Operated Control	5	0	-
PB	5	0	-
DENA + PB	5	0	-
AsIII (160ppm)	5	0	-
ASV (160ppm)	5	0	-
PH, Control	5	0	-
PH, DENA	5	3	++
PH, PB	5	0	-
PH, AsIII (160ppm)	5	1	++
PH, AsV (160ppm)	5	1	+++
PH, DMA (80ppm)	4	0	-
PH, MA (800ppm)	4	2	++
PH, DENA + PB	5	2	+
PH, DENA + AsIII (80ppm)	5	1	++
PH, DENA + AsV (80ppm)	5	1	+++
PH, DENA + AsIII (160ppm)	5	4	++
PH, DENA + AsV (160ppm)	5	4	++
PH, DENA + DMA (80ppm)	9	1	+
PH, DENA + MA (800ppm)	7	0	-

Table 6-2. Organ-Body Weight Ratios of DENA-Initiated Wistar Rats Chronically Exposed to Inorganic Arsenic in the Drinking Water for 7 Weeks.

Experimental	Thymus	Spleen	Kidney	Adrenal	Liver
S-O Control	.248±.013	.231±.015	.359±.015	.010 ± .002	2.65±.09
PB	.185±.031	.237±.028	.425±.011	.009 ± .002	3.27±.11
DENA	.188±.025	.215±.027	.358±.023	.011 ± .003	2.51±.09
DENA, PB	.269±.020	.217±.005	.402±.008	.017 ± .001	3.46±.05
PH, Control	.197±.022	.225±.023	.391±.015	.011 ± .003	2.46±.12
PH, DENA	.153±.011	.222±.008	.365±.022	.008 ± .001	2.51±.08
PH, PB	.177±.021	.247±.023	.384±.013	.010 ± .001	3.16±.12
PH, AsIII	.177±.007	.222±.015	.442±.009	.008 ± .001	3.10±.15
PH, AsV	.179±.021	.227±.007	.511±.058	.008 ± .001	2.63±.07
PH, DENA, PB	.208±.023	.245±.017	.417±.03	.012 ± .002	3.47±.15
PH,DENA,AsIII(80ppm)	.195±.015	.227±.003	.433±.015	.008 ± .001	2.83±.08
PH,DENA,AsV (80ppm)	.180±.011	.190±.009	.411±.009	.007 ± .001	2.45±.09
PH,DENA,AsIII(160ppm)	.182±.007	.228±.014	.427±.013	.007 ± .001	2.75±.25
PH,DENA,AsV(160ppm)	.153±.021	.203±.013	.425±.010	.007 ± .001	2.82±.10

membrane-bound ATPase activity.

The effects of the organic arsenics on GGTase activity were not dramatic both histochemically and biochemically. Histochemically there were no GGTase positive foci present.

Table 6-3. Effect on Hepatic GGTase from DENA-Initiated Wistar Rats Exposed to Inorganic Arsenics in the Drinking Water for 7 Weeks.

Experimental Condition	GGTase Activity	
	Histochemical	Biochemical
Control	0.3 ± 0.21 (6)	0.47 ± 0.07 (6)
Sham Operated Control	1.5 ± 0.33 (14)	0.79 ± 0.20 (14)
PB	1.6 ± 0.40 (5)	0.83 ± 0.20 (5)
DENA	3.0 ± 0.45 (5)	2.47 ± 0.80 (5)
DENA + PB	3.3 ± 0.48 (4)	4.56 ± 1.79 (4)
PH, Control	1.5 ± 0.31 (4)	0.75 ± 0.40 (11)
PH, DENA	1.6 ± 0.24 (9)	0.70 ± 0.19 (9)
PH, PB	2.2 ± 0.32 (9)	0.95 ± 0.20 (9)
PH, AsIII (160ppm)	3.2 ± 0.32 (9)	3.28 ± 0.81 (9)
PH, AsV (160ppm)	3.4 ± 0.28 (11)	3.10 ± 0.58 (11)
PH, MA (800ppm)	0.5 ± 0.29 (4)	0.30 ± 0.07 (4)
PH, DMA (80ppm)	2.5 ± 0.50 (4)	0.87 ± 0.44 (4)
PH, DENA + PB	3.1 ± 0.55 (7)	3.44 ± 1.34 (7)
PH, DENA + AsIII (80ppm)	3.2 ± 0.49 (5)	2.36 ± 0.91 (5)
PH, DENA + AsV (80ppm)	2.2 ± 0.20 (5)	0.68 ± 0.09 (5)
PH, DENA + AsIII (160ppm)	3.6 ± 0.25 (5)	5.55 ± 1.30 (5)
PH, DENA + AsV (160ppm)	3.6 ± 0.20 (7)	4.10 ± 0.88 (7)
PH, DENA + MA (800ppm)	2.6 ± 0.37 (7)	1.39 ± 0.33 (7)
PH, DENA + DMA (80ppm)	2.6 ± 0.13 (9)	1.84 ± 0.48 (9)

The data are expressed as mean values ± std error of the means.

( ) denotes the number of animals in each treatment group.

Light Microscopic Examination

The H & E stained liver sections were examined for hyperplastic nodule, neoplastic nodule and hepatocellular carcinoma. The results of the light microscopic examination of these tissue sections did not demonstrate any significant presence of the above-mentioned lesions.

DISCUSSION

Although previous studies showed significant increase in GGTase activity in the liver after arsenic treatment, the results of this study showed very clearly that identification of discrete foci of GGTase activity in livers from DENA-initiated animals treated with arsenic as a potential promoter was difficult. Foci of GGTase activity were difficult to identify because of the large, diffuse background activity which AsIII and AsV characteristically cause.

On the other hand, we did not have any difficulty demonstrating discrete areas of GGTase activity with PB which was our positive control. Thus, we do not feel that our negative results reflected any technical difficulties

because we were able to reproduce the PB effects in our laboratory.

Our conclusion was that we had to develop a second marker system to use in combination with the GGTase activity to identify the foci. We have decided to develop the membrane ATPase histochemical assay as the second marker system. In this system there is a deficiency of enzyme activity, therefore, we will be able to match up an area of increased GGTase activity with an area of deficiency of ATPase activity. Thus, this would be the criteria for potential preneoplastic lesions in the liver.

SECTION 7

PROMOTER POTENTIAL OF ARSENIC IN PARTIALLY HEPATECTOMIZED DENA-INITIATED RATS: II. The Effects in the Liver of Sprague-Dawley Rats Treated With Arsenic in the Drinking Water for 7 Weeks.

INTRODUCTION

Since it appeared that the GGTase activity in the Wistar rats was not as good as we anticipated, in terms of foci and intensity, we thought it might have been due to the strain of rats. Therefore, we decided to repeat our experiments in the Sprague-Dawley rats which were originally used for these initiation-promotion studies by other investigators. The Fischer rats were also used.

MATERIAL AND METHODS

Same as in Section 6.

RESULTS

The results of these experiments with the Sprague-Dawley rats were qualitatively the same as that for the Wistar strain. But the Wistar rats appeared to be more sensitive to arsenic treatment than were the Sprague-Dawley strain. GGTase activity was found to be greater in the Wistar rats, whereas the Sprague-Dawley was more susceptible to hyperplastic nodules which are nonspecific and are very often reversible responses to liver trauma.

Table 7-1. Hyperplastic Nodules in DENA-Initiated Sprague-Dawley Rat Livers Chronically Exposed to Inorganic Arsenics in Drinking Water for 7 Weeks.

Experimental Condition	n	No. Animals with Nodules	Degree of Nodule Formation
Sham Operated Control	6	0	-
PB	5	0	-
DENA	5	0	-
DENA + PB	4	1	++++
PH, Control	7	0	-
PH, PB	4	0	-
PH, DENA	5	0	-
PH, DENA + PB	9	6	+
PH, DENA + AsIII (160ppm)	5	1	+
PH, DEN + AsV (160ppm)	5	2	+

Table 7-2. Organ-Body Weight Ratios of DENA-Initiated Sprague-Dawley Rats Chronically Exposed to Inorganic Arsenics in the Drinking Water for 7 Weeks.

Experimental Condition	Thymus	Spleen	Kidney	Adrenal	Liver
S-O Control	.150 ±.009	.211 ±.016	.387 ±.016	.007 ± .0005	2.79 ± .04
PB	.172 ±.020	.219 ±.009	.395 ±.009	.006 ± .0004	3.41 ± .07
DENA	.143 ±.007	.194 ±.009	.363 ±.012	.006 ± .0003	2.57 ± .04
DENA, PB	.161 ±.008	.232 ±.013	.371 ±.008	.007 ± .0003	3.32 ± .10
PH, Control	.161 ±.010	.224 ±.005	.383 ±.008	.007 ± .0003	2.60 ± .03
PH, PB	.176 ±.008	.220 ±.017	.382 ±.013	.007 ± .0007	3.33 ± .08
PH, DENA	.174 ±.008	.229 ±.009	.349 ± .009	.007 ± .0005	2.58 ± .08
PH, DENA, PB	.165 ±.018	.245 ±.013	.376 ±.004	.007 ± .0001	3.71 ± .05
PH, DENA, AsIII	.166 ±.008	.266 ±.016	.478 ±.011	.008 ± .0014	2.72 ± .08
PH, DENA, AsV	.136 ±.015	.204 ±.017	.505 ±.078	.006 ± .0007	2.43 ± .21

The data are expressed as the mean ratio ± std error of the means.

Table 7-3. Effect on GGTase Activity in Livers from DENA-Initiated Sprague-Dawley Rats Chronically Exposed to Inorganic Arsenics in The Drinking Water for 7 Weeks.

Experimental Condition	n	GGTase Activity	
		Histochemical	Biochemical
Sham Operated Control	5	0.5 ± 0.26	0.36 ± 0.00
PB	5	0.8 ± 0.58	0.43 ± 0.22
DENA	6	0.8 ± 0.33	0.27 ± 0.04
DENA + PB	4	2.0 ± 0.35	0.83 ± 0.43
PH, Control	7	1.1 ± 0.37	0.34 ± 0.06
PH, PB	4	2.0 ± 0.57	0.61 ± 0.28
PH, DENA	5	2.2 ± 0.36	0.05 ± 0.16
PH, DENA + PB	9	2.9 ± 0.99	2.42 ± 0.13
PH, DENA + ASIII (160ppm)	5	3.2 ± 0.20	2.94 ± 0.98
PH, DENA + AsV (160ppm)	5	3.4 ± 0.22	4.08 ± 0.93

The data are expressed as mean values ± std error of the means.

DISCUSSION

Our conclusions were that we should continue our studies in the Wistar rats because it appeared that this strain responded better to arsenic treatment, that is, there appeared to be a greater effect on causing cellular changes in the liver with arsenic. On the other hand, the PB effects appeared to be more pronounced in the Sprague-Dawley strain, in particular the hyperplastic nodule effect.

Although further studies are required, the results from these experiments suggested that there were strain differences in tissue response to a particular toxicological substance.

SECTION 8

PROMOTER POTENTIAL OF ARSENIC IN PARTIALLY HEPATECTOMIZED DENA-INITIATED RATS: III. The Effects in the Liver of Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 25 and 43 Weeks.

INTRODUCTION

Section 6 experiments were extended to longer time periods. In addition sham-operated animals were included in this study and treated similarly as the partially hepatectomized animals in the promotion experiments. The results from these animals will determine whether the partial hepatectomy was essential for the experimental design of this study.

MATERIALS AND METHODS

The procedures used in this study was the same as those employed in Section 6 except that the experiments continued to longer time periods, and sham-operated animals were included as control treatment groups for the promotion experiments.

RESULTS

Sham-Operated Controls

It was evident from these experiments that very few cellular alterations were observed in these animals (Tables 8-1 to 8-3) regardless of the treatment. It is true that the number of animals in several of the treatment groups were relatively small, therefore, definite conclusions cannot be made from these experiments. But these results are not surprising in lieu of the presumed basis for the 2 stage initiation-promotion model is the partial hepatectomy.

Partially Hepatectomized Animals

GGTase Activity--

The histochemical and biochemical assays of GGTase activity are shown in Tables 8-4 and 8-5. The effects observed increased as the length of treatment increased with both PB and the inorganic arsenics. The effects of the organic arsenics did not increase but remained the same in the two time periods. When compared to the 7 week treatment (Section 6), there was no increase with MA and a very small increase with DMA.

Preliminary histochemical ATPase assays in the liver tissue appeared to be promising, especially in those livers promoted by PB. We found many foci of GGTase activity which matched up with ATPase deficient areas. However, for

arsenic treatment these mutual areas were much more difficult to identify for much the same reasons as discussed previously.

Table 8-1. The Formation of Basophilic Foci in Livers of Sham-Operated DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 25 and 43 Weeks.

Treatment	No. of Animals With Basophilic Foci/Treatment Group	
	25 Weeks	43 Weeks
Control	0/10	0/13
PB	0/5	0/3
DENA	0/3	3/9
DENA + AsIII	4/7	0/2
DENA + AsV	0/5	1/5
DENA + DMA	1/3	0/4
DENA + MA	3/9	1/6
DENA + PB	0/5	2/5

Table 8-2. The Formation of Neoplastic Nodules in Livers of Sham-Operated DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 25 and 43 Weeks.

Treatment	No. of Animals With Neoplastic Nodule/Treatment Group	
	25 Weeks	43 Weeks
Control	0/10	0/13
PB	0/5	0/3
DENA	0/3	0/9
DENA + AsIII	0/7	0/2
DENA + AsV	0/5	0/5
DENA + DMA	0/3	0/4
DENA + MA	0/9	0/6
DENA + PB	0/5	0/5

Table 8-3. The Formation of Hepatocellular Carcinoma in Livers of Sham-Operated DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 25 and 43 Weeks.

Treatment Group	No. of Animals With Hepatocellular Carcinoma/Treatment	
	25 Weeks	43 Weeks
Control	0/10	0/13
PB	0/5	0/3
DENA	0/3	0/9
DENA + AsIII	0/7	0/2
DENA + AsV	0/5	1/5
DENA + DMA	0/3	0/4
DENA + MA	0/9	0/6
DENA + PB	0/5	0/5

Table 8-4. Effect on Hepatic GGTase from DENA-Initiated Wistar Rats Exposed to Inorganic Arsenics in the Drinking Water for 25 Weeks.

Experimental Condition	GGTase Activity	
	Histochemical	Biochemical
Control H_2O	1.2 ± 0.33 (14)	0.52 ± 0.17 (14)
Sham Operated Control	1.8 ± 0.36 (10)	1.06 ± 0.33 (8)
PB	1.0 ± 0.42 (8)	0.69 ± 0.22 (8)
DENA	1.8 ± 0.42 (5)	1.38 ± 0.58 (5)
DENA + PB	2.8 ± 0.25 (8)	2.26 ± 0.74 (8)
AsIII (160ppm)	3.0 ± 0.57 (3)	4.31 ± 2.91 (3)
AsV (160ppm)	2.8 ± 0.85 (4)	2.83 ± 1.82 (4)
PH, Control	1.3 ± 0.27 (15)	0.91 ± 0.50 (15)
PH, DENA	2.6 ± 0.41 (9)	1.96 ± 0.50 (9)
PH, PB	2.3 ± 0.42 (10)	2.30 ± 0.65 (10)
PH, AsIII (160ppm)	3.6 ± 0.34 (10)	3.93 ± 0.86 (10)
PH, AsV (160ppm)	3.0 ± 0.45 (6)	2.99 ± 0.84 (6)
PH, MA (800ppm)	1.6 ± 0.48 (7)	0.38 ± 0.12 (7)
PH, DMA (80ppm)	2.9 ± 0.30 (8)	1.35 ± 0.25 (8)
PH, DENA + PB	3.5 ± 0.31 (10)	4.14 ± 1.11 (10)
PH, DENA + AIII (160ppm)	3.5 ± 0.34 (10)	4.51 ± 0.94 (10)
PH, DENA + AsV (160ppm)	3.4 ± 0.37 (10)	3.94 ± 0.77 (10)
PH, DENA + MA (800ppm)	2.6 ± 0.27 (10)	1.41 ± 0.28 (10)
PH, DENA + DMA (80ppm)	3.1 ± 0.40 (7)	2.28 ± 0.63 (7)

Histology by H & E--

Very few basophilic foci were observed in animals treated with arsenics and PB alone as shown in Table 8-6. However, formation of basophilic foci with DENA treatment was significant. These effects appeared to be maximal by 25 weeks of treatment. Therefore, any additional effects by the arsenics or PB could not be observed.

In the formation of neoplastic nodules as shown in Table 8-7, no effects were observed with the arsenics or PB alone. However, with DENA treatment after 43 weeks, the number of nodules were increased over the 25 week treatment. The arsenics did not increase neoplastic nodule formation in DENA-initiated livers. However, PB, a known promoter, increased significantly the number of neoplastic nodules in DENA-initiated animals.

Of all the animals observed in this study we found only two animals in which hepatocellular carcinoma was found (Table 8-8). Interestingly the adenocarcinomas were found in animals from the same treatment group, namely, the DENA initiated DMA treated animals. However, the results were not significant.

Table 8-5. Effect on Hepatic GGTase from DENA-Initiated Wistar Rats Exposed to Inorganic Arsenics in the Drinking Water for 43 Weeks.

Experimental Condition	GGTase Activity	
	Histochemical	Biochemical
Control H_2O	1.4 ± 0.25 (14)	0.63 ± 0.15 (14)
Sham Operated Control	2.2 ± 0.23 (13)	0.78 ± 0.16 (13)
PB	1.6 ± 0.25 (5)	0.86 ± 0.24 (5)
DENA + PB	3.0 ± 0.00 (2)	3.00 ± 0.71 (2)
AsIII (160ppm)	3.2 ± 0.26 (13)	5.22 ± 0.94 (13)
AsV (160ppm)	3.1 ± 0.21 (14)	4.29 ± 0.92 (14)
PH, Control	1.9 ± 0.30 (8)	1.22 ± 0.47 (8)
PH, DENA	2.0 ± 0.24 (9)	1.26 ± 0.26 (9)
PH, PB	3.3 ± 0.86 (4)	4.32 ± 2.08 (4)
PH, AsIII (160ppm)	3.8 ± 0.25 (4)	7.52 ± 2.13 (4)
PH, AsV (160ppm)	3.6 ± 0.32 (8)	5.97 ± 0.98 (8)
PH, MA (800ppm)	1.5 ± 0.50 (2)	0.63 ± 0.35 (2)
PH, DMA (80ppm)	1.5 ± 0.50 (2)	0.60 ± 0.40 (2)
PH, DENA + PB	3.7 ± 0.17 (13)	6.38 ± 1.05 (13)
PH, DENA + AIII (160ppm)	3.6 ± 0.18 (8)	6.91 ± 1.57 (8)
PH, DENA + AsV (160ppm)	3.7 ± 0.19 (12)	6.47 ± 1.07 (12)
PH, DENA + MA (800ppm)	2.2 ± 0.49 (5)	1.61 ± 0.63 (5)
PH, DENA + DMA (80ppm)	2.5 ± 0.24 (13)	2.73 ± 0.55 (13)

Table 8-6. The Formation of Basophilic Foci in Livers of Partially Hepatectomized DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 25 and 43 Weeks.

Treatment	No. of Animals With Basophilic Foci/Treatment Group	
	25 Weeks	43 Weeks
Control	0/15	0/8
AsIII	2/10	0/4
AsV	0/6	0/8
DMA	2/8	2/2
MA	0/7	0/2
PB	2/10	1/4
DENA	2/10	1/4
DENA + AsIII	8/10	8/8
DENA + AsV	8/10	11/12
DENA + DMA	7/7	10/13
DENA + MA	8/10	5/5
DENA + PB	10/10	11/13

Table 8-7. The Formation of Neoplastic Nodules in Livers of Partially Hepatectomized DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 25 and 43 Weeks.

Treatment	No. of Animals With Neoplastic Nodule/Treated Group	
	25 Weeks	43 Weeks
Control	0/15	0/8
AsIII	0/10	0/4
AsV	0/6	0/8
DMA	0/8	0/2
MA	0/7	0/2
PB	0/10	0/4
DENA	1/9	3/9
DENA + AsIII	0/10	3/8
DENA + AsV	0/10	2/12
DENA + DMA	1/7	3/13
DENA + MA	0/10	0/5
DENA + PB	2/10	8/13

Table 8-8. The Formation of Hepatocellular Carcinoma in Livers of Partially Hepatectomized DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 25 and 43 Weeks.

Treatment	No. of Animals With Hepatocellular Carcinoma/Treatment Group	
	25 Weeks	43 Weeks
Control	0/15	0/8
AsIII	0/10	0/4
AsV	0/6	0/8
DMA	0/8	0/2
MA	0/7	0/2
PB	0/10	0/4
DENA	0/9	0/9
DENA + AsIII	0/10	0/8
DENA + AsV	0/10	0/12
DENA + DMA	0/7	2/13
DENA + MA	0/10	0/5
DENA + PB	0/10	0/13

DISCUSSION

Although the results from this study were not revealing, there might be some tentative conclusions to be made. First, the initiator, DENA, did not have any dramatic effect on alteration of liver cells unless partial hepatectomy was a component of the experimental design, at least during the time course of this investigation. Thus, PH might be a requisite for the DENA-initiation effects and/or accelerates the process such that the effect is observed at an earlier time frame.

The criterion of using GGTase activity as an index for potential discrete areas of preneoplastic lesions was found to be difficult to use. The use of a double enzyme marker system, that is, the presence of a focus of GGTase activity and the absence of ATPase activity appeared not to be useful in arsenic treated animals because of the large, diffuse background activity but probably would be very useful in PB-promotion studies.

Although basophilic foci formation was associated with DENA-initiation and were observed prior to neoplastic nodules, it is questionable whether these observations will indicate any correlation with the eventual formation of an adenocarcinoma in the liver. However, we feel that liver sections should be evaluated for the presence of neoplastic nodules to determine whether any correlation exits with the eventual apppearance of hepatocellular carcinomas with arsenic effects. There does not appear to be any correlation with PB effects.

Finally, it is clear that no conclusions can be made with respect to

arsenic effects, although there is an indication that DMA might promote the formation of hepatocellular carcinoma in the liver. Longer treatment periods are necessary because the arsenic doses employed in these studies were at the MTD levels and can not be increased. The other possibility is to increase the initiating dose of DENA so that the effects might be observed at an earlier time.

SECTION 9

PROMOTER POTENTIAL OF ARSENIC IN PARTIALLY HEPATECTOMIZED DENA-INITIATED RATS: IV. Renal Tubular Tumorigenesis With Chronic Arsenic Treatment in the Drinking Water of Wistar Rats for 7, 25 and 43 Weeks.

INTRODUCTION

During the course of these promoter experiments, several DENA-initiated arsenic treated animals died after approximately ten months of treatment, just two months prior to their scheduled sacrifice. The deaths were very unusual because we had not lost any animals up to this point in time except for during the toxicity studies. In addition we had also sacrificed some DENA-initiated arsenic treated animals at 25 weeks and much to our surprise we found kidney tumors in these animals. Therefore, we made a decision to sacrifice the 12 month animals at 43 weeks, making the assumption that the animals had died from the kidney tumors. After sacrificing more animals at 24 and 43 weeks, it became apparent that our assumption was correct.

MATERIALS AND METHODS

Animals

The animals in this study are those described in Sections 6 and 8. The water intake for each cage was monitored daily. From this data a daily doses for the arsenics were calculated. At the scheduled sacrifice days, both kidneys were removed from each animal, weighed and processed for histochemical, biochemical and histological examinations.

GGTase Assays

Kidney tissues were prepared and histochemical and biochemical assays were performed as previously described.

Histology

Kidney tissues were prepared as described for liver tissues in Section 6.

Histological Evaluation

The kidney sections were evaluated for atypical hyperplasia, renal adenoma and adenocarcinoma as described by Burek (1978) and by personal communication with Dr. Gary Boorman at N.I.E.H.S. We also developed a quality assurance procedure in consultation with Dr. Mike Stedman of Pathology Associates, Inc.

Statistical Analysis

All quantal data were evaluated by Chi Square and all other quantitative data were evaluated by Student t test.

RESULTS

Calculated Daily Intake of Arsenic

The calculated daily doses of the various arsenics under the different experimental conditions of this study are shown in Table 9-1. The data show that the animals treated with the inorganic arsenics drank less water than the controls, AsIII approximately 32% regardless of whether the animals were DENA-initiated and AsV approximately 12% in the absence of and 22% in the presence of DENA-initiation.

DMA treated animals drank about the same as the controls, perhaps slightly more than controls with DENA-initiation. But MA increased water intake in both conditions, 87% and 54% respectively. Thus, there was a tendency for organic arsenics to stimulate drinking behavior, particularly MA.

Due to these differences in drinking behavior, the actual daily doses of the different arsenics was not reflected by the concentrations administered to the animals in the drinking water. Despite these differences the daily dose of DMA was still slightly less than that of AsIII and AsV, whereas the dose of MA was 10-12 fold greater.

Table 9-1. The Dose of Elemental Arsenic Ingested Daily by Partially Hepatectomized, DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water.

Experimental Condition	Concentration of Elemental As (in ppm)	Mean H_2O Intake/day (in ml±s.e.m.)	Mean Elemental As Ingested/day (in mg ± s.e.m.)
Control	-	39.3 ± 4.0	-
AsIII	160	26.8 ± 3.0	4.4 ± 0.5
AsV	160	34.7 ± 0.8	5.6 ± 0.2
DMA	80	41.9 ± 0.9	3.4 ± 0.1
MA	800	73.4 ± 1.3	58.6 ± 1.0
DENA	-	37.9 ± 4.5	-
DENA + AsIII	160	25.3 ± 2.3	4.1 ± 0.4
DENA + AsV	160	29.5 ± 0.8	4.7 ± 0.1
DENA + DMA	80	46.9 ± 6.1	3.8 ± 0.5
DENA + MA	800	58.3 ± 2.8	46.6 ± 2.2

Sham-Operated Animals

The partial hepatectomy component of the experimental design of this study required that sham-operated animals be treated similarly in order to determine whether PH was a requisite component for or was necessary to accelerate the possible carcinogenesis process in DENA-initiated animals.

Atypical hyperplasia of the renal tubular cells which has been suggested to be a preneoplastic lesion in the kidney was somewhat variable in these animals as shown in Table 9-2. No definite trend was established.

With respect to tumor formation in the kidney (Table 9-3), no tumors were observed with 25 weeks of arsenic or PB treatment. After 43 weeks of arsenic treatment, only one tumor was observed in the DMA promoted animals, but 2 of 6 animals had tumors in the MA promoted animals.

Table 9-2. The Atypical Hyperplasia Effect in the Kidney of Sham-Operated DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 7, 25 and 43 Weeks.

Treatment	No. of Animals With Atypical Hyperplasia/Treatment Group		
	7 Weeks	25 Weeks	43 Weeks
Control	2/14	0/10	0/13
PB	1/5	0/5	0/3
DENA	0/10	0/3	2/9
DENA + AsIII	2/5	4/7	1/2
DENA + AsV	1/4	3/5	1/5
DENA + DMA	3/4	0/3	3/4
DENA + MA	1/3	7/9	5/6
DENA + PB	0/2	1/5	1/7

Table 9-3. The Tumor Effect in the Kidneys from Sham-Operated DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 7, 25 and 43 Weeks.

Treatment	No. of Animals With Renal Tumors/Treatment Group		
	7 Weeks	25 Weeks	43 Weeks
Control	0/14	0/10	0/13
PB	0/5	0/5	0/3
DENA	0/10	0/3	0/9
DENA + AsIII	0/5	0/7	0/2
DENA + AsV	0/4	0/5	0/5
DENA + DMA	0/4	0/3	1/4
DENA + MA	0/3	0/9	2/6
DENA + PB	0/2	0/5	0/7

Partial Hepatectomized Animals

Atypical hyperplasia was observed in nearly all of the treatment groups, including animals which were not DENA-initiated as shown in Table 9-4. Increasing the arsenic or PB treatment period from 25 to 43 weeks generally increased the incidence of atypical hyperplasia per treatment group.

The number of renal tubular tumors observed in this study is shown in Table 9-5. No tumors were observed in animals which were not DENA-initiated. Thus, in this study there was no experimental evidence that arsenic alone could cause tumorigenesis in the kidney. No tumors were observed in any of the treatment groups after 7 weeks of treatment. However, AsIII significantly increased the incidence of tumor formation in DENA-initiated animals after 25 weeks of treatment. At 43 weeks of treatment, most of the DENA-initiated animals exhibited a high incidence of renal tubular tumorigenesis, however, the effects were not statistically significant however, the effects were not statistically significant when compared to the DENA controls. This was due primarily to the high incidence of tumors observed with DENA treatment alone. Thus, the 25 week treatment data supported the hypothesis that AsIII may act as a promoter for DENA.

The effect of the arsenics on the thymus gland is shown in Table 9-6. The results showed that AsIII caused a significant decrease in the thymus weight after 25 weeks of treatment. AsIII also decreased the thymus weight in the DENA-initiated rats significantly. All other comparisons were not significant.

Table 9-4. The Atypical Hyperplasia Effect in the Kidney of Partially Hepatectomized DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 7, 25 and 43 Weeks.

Treatment	No. of Animals With Atypical Hyperplasia/Treatment Group		
	7 Weeks	25 Weeks	43 Weeks
Control	0/8	1/15	2/8
AsIII	4/7	2/10	1/4
AsV	7/11	1/6	4/8
DMA	0/4	0/8	1/2
MA	0/4	0/7	0/2
PB	0/9	2/10	0/3
DENA	1/7	4/9	6/9
DENA + AsIII	1/5	9/10	8/8
DENA + AsV	5/7	5/10	11/12
DENA + DMA	2/8	1/7	11/13
DENA + MA	0/1	4/10	0/5
DENA + PB	0/7	3/10	6/13

Table 9-5. The Tumorigenic Effect in the Kidney of Partially Hepatectomized DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 7, 25 and 43 Weeks.

Treatment	No. of Animals With Renal Tumors/Treatment Group		
	7 Weeks	25 Weeks	43 Weeks
Control	0/8	0/15	0/8
AsIII	0/7	0/10	0/4
AsV	0/11	0/6	0/8
DMA	0/4	0/8	0/2
MA	0/4	0/7	0/2
PB	0/9	0/10	0/3
DENA	0/7	2/9	6/9
DENA + AsIII	0/5	7/10*	8/8
DENA + AsV	0/7	3/10	9/12
DENA + DMA	0/8	3/7	9/13
DENA + MA	0/1	4/10	0/5
DENA + PB	0/7	1/10	8/13

* $P < 0.05$ when compared with the DENA treatment group by Chi Square.

Table 9-6. The Effect on the Thymus Gland from DENA-Initiated Wistar Rats Chronically Treated With Arsenic in the Drinking Water for 25 Weeks.

Treatment	Mean Wet Weight of the Thymus (in mg ± s.e.m.)
Control	367.3 ± 29.9
AsIII	274.7 ± 18.3 **
AsV	292.3 ± 37.5
DMA	341.0 ± 22.8
MA	321.4 ± 31.8
DENA	318.3 ± 24.1
DENA + AsIII	226.8 ± 22.1 *
DENA + AsV	269.2 ± 20.4
DENA + DMA	345.9 ± 36.7
DENA + MA	325.5 ± 37.6

* P value <0.05 when compared to DENA control
** P value <0.01 when compared to control

DISCUSSION

From the water intake monitoring data, it was shown that AsIII and to a lesser extent AsV inhibited the drinking behavior of the partially hepatectomized animals at the 160ppm concentration these arsenics were present in the drinking water. DENA-initiation did not have any effect on the AsIII

inhibition and increased the inhibition effect of AsV from 12% to 22%. Comparing data from subsequent experiments in which non-hepatectomized animals were chronically treated with AsIII and AsV these results showed somewhat greater inhibitions, although the actual volumes which were drunk daily were quite similar. Therefore, the partial hepatectomy was probably not a determining factor in this effect. The other possibilities are the bad taste of these anions in solution or gastric irritation, inhibiting drinking behavior, or these anions have a direct effect on the hypothalamic thirst center to inhibit drinking. The more likely effect would be the former rather than the latter.

DMA appeared to have the least effect on the drinking behavior of these animals. With DENA-initiation there was a slight increase in water intake daily, but it did not significantly increase the mean daily dose of elemental arsenic. However, MA significantly increased the drinking behavior by as much as 87% in the absence of and 54% in the presence of DENA-initiation. As with the inorganic arsenics there was not any qualitative difference in this effect in non-hepatectomized animals. Thus, partial hepatectomy does not appear to be a determining factor. It is not clear whether this effect is due to the taste of this anion to increase drinking behavior, or it is a direct effect on the hypothalamus to cause these animals to drink more water.

It was not clear whether atypical hyperplasia was a preneoplastic lesion in our experiments because we observed this pathological lesion in the kidney of nearly all of our sham-operated DENA-initiated treatment groups after 43 weeks of arsenic treatment but observed only a total of 3 animals with tumors. In the partially hepatectomized animals we made a similar observation with respect to atypical hyperplasia. In nearly all of the inorganic arsenic treated groups of animals DENA-initiated or not showed the presence of atypical hyperplasia in the kidney. With the organic arsenic treatment there appeared to be less incidence of atypical hyperplasia in the treatment groups. However, the number of tumors observed in the partially hepatectomized animals *in toto* was vastly increased. This would suggest that this increase was probably due to the partial hepatectomy which enhanced the DENA-initiation. This did not answer the question of whether the presence of atypical hyperplasia might indicate a preneoplastic lesion.

It was statistically significant that AsIII promoted DENA-initiated tumors in the kidney. The tumors were renal tubular adenomas or adenocarcinomas located in the cortical areas of the kidney. We did not find any tumors in the medullary region. Very often the tumors were bilateral, and this is probably the reason why we observed animals dying initially.

It is not clear what the mechanism is for the promotion effect of AsIII we observed in this study. However, the observation that the thymus weight was significantly decreased by AsIII might be experimental evidence to involve the immune system. It is generally accepted that a decrease in thymus function results in a decrease in the immune response. And immunosuppression has been suggested to be a contributory factor in some cancers in humans.

Finally, there are some important questions which have to be asked. What is the role of partial hepatectomy in these experiments, since this surgical procedure is used in studying hepatectomy in these experiments, since this surgical procedure is used in studying hepatocarcinogenesis not renal carcinogenesis? Did the promotion effect of AsIII observed in this study require that partial hepatectomy be a necessary requisite? Or did the partial hepatectomy only accelerate the tumorigenesis in the kidney? Is there a difference in the metabolism of arsenics in the kidney as compared with the liver which is important for renal tumorigenesis?

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